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Sir:

Applicant has claimed priority under 35 U.S.C. § 119 to European Patent Application No. 98118938.4, filed on October 7, 1998. In support of this claim, a certified copy of this application is submitted herewith.

No fee or certification is believed to be due for this submission. Should any fee be required, however, please charge such fee to Winston & Strawn LLP Deposit Account No. 50-1814.

Respectfully submitted,

4/1/04
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Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

98118938.4

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

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Novel process for the cryo-preservation of plants

The present invention pertains to an improved process for the cryo-preservation of plants, especially for the direct cryo-preservation of plant tissues.

With the upcome of in vitro culture techniques the possibilities for the collection, ex situ conservation and the directed genetic exchange of plant species that produce no or merely short-lived (recalcitrant) seeds or that are vegetatively propagated have extensively increased in number.

Said in vitro techniques also offer the possibility of eliminating pathogens and thus preserving and exchanging germplasm in a disease-free condition. In order to preserve genetic plant material the objective material has brought into a condition of being stored for a predetermined period of time, with the preserved tissue also being rendered capable to regrow to a plant at a later stage. By proceeding accordingly the risk of contaminating the plant material by various plant pathogens might be substantially avoided .

Preservation techniques that have been developed so far include e.g. retarding the growth of the particular plant or preserving plants at a particular stage of its development at low temperatures, such as cryo-preservation in liquid nitrogen at -196°C for long term storage. Retardation of plant growth is often used for recalcitrant species, the seeds of which cannot be stored. Examples for such species are e.g. many tropical plants, such as coffee or cocoa. This option does, however, only provide a limited period of time for storage, since the plant will eventually grow and occasionally change its genetic material. Moreover, this practice is very expensive and time-consuming and requires sophisticated equipment.

It is widely acknowledged that for long-term storage of genetic plant material, such as of germplasm of vegetatively propagated species and of recalcitrant species, cryo-preservation is the desired option. This process involves storage at ultra-low temperatures, usually that of liquid nitrogen (-196°C), at which temperature cell division and metabolic processes come to a stop and the plant material can thus be stored without modification or alteration for prolonged periods of time. Other advantages of this technique reside in that cryo-preserved material requires only

limited space, is protected from contamination and needs only moderate maintenance efforts.

Processes for the long-term preservation of plants have been developed only for a restricted number of plant species, wherein the original plant or parts thereof are treated in a number of steps so as to obtain explants in developmental late stages including zygotic and somatic embryos, meristems etc., which are found to be eventually capable to grow to a new plant after cryo-preservation. However, it is acknowledged that the longer the duration of the in vitro culture before cryo-preservation, that is the higher the number of pretreatment steps, the higher the risk of somaclonal variation.

It would therefore be desirable to limit the steps in culture before cryo-preservation to as few as possible.

Apart from the drawbacks involved in subjecting the plant derived tissue to a number of pretreatment steps to arrive at the desired explant tissue that is finally capable to grow to a new plant, some developmental stages of the plant, such as e.g. zygotic embryos, are found to be merely usable for pure lines of self-pollinated species thus limiting the applicabilty of such procedures. For other species merely vegetative tissues may be used, such as meristems, shoot tips, regenerating tissues or somatic embryos. Consequently, the choice of the specific material for storage will depend on a variety of parameters, such as the plant species, the techniques available, the genetic structure of the population being stored and the available germplasm.

Finally, in order to limit the risk of somaclonal variation, the preserved tissue should regenerate from the cryo-preserved material as directly as possible into plantlets. Therefore, calli and/or embryogenic tissues are less desirable than somatic embryos, which could directly regrow without any secondary embryogenesis or callogenesis.

It is therefore a need in the art to provide a method, with which a variety of different plant species may be stored without involving the laborious steps of culturing plant material until a late developmental stage of the plant may be obtained, that is capable to grow to a full plant. Accordingly an object of the present invention is to provide an improved method, with which a variety of different plants may be

processed for storage at low temperatures without involving complicated steps to obtain a plant tissue suitable for regeneration to a new plant.

This object has been solved by a process comprising the steps of dehydrating, pre-freezing and cryo-freezing plant derived tissue, wherein the plant tissue to be cryo-preserved is a primary explant that has been subjected to an induction treatment for regeneration.

In a preferred embodiment the plant tissue utilized in the process according to the present invention is a primary explant capable to regenerate buds.

In another preferred embodiment the process for the cryo-preservation of the present invention comprises using a plant tissue capable to regenerate embryos.

It has been found that the process of the present invention may be applied to any plant / plant material known in the art. It will, however, be mainly applied to plant species, which cannot be stored in an easy way, such as e.g. semi-recalcitrant plant species or recalcitrant plant species. In another preferred embodiment the plant from which the tissues to be processed for cryo-preservation may be derived, is a plant of the genus Theobroma cacao L., that is cacao, or of the genus Coffea Canephora or Coffea arabica L., i.e. coffe, or of the genus Daucus carota, e.g. carrots.

The induction of the plant tissues may be achieved by cultivating the plant or a particular part thereof, such as e.g. part of a leaf, part of a stem, a flower bud or hypocotyl segments that have been cut off the plant.

This plant derived tissue may then be transferred to an induction buffer, such as e.g. those described in Driver & Kuniyuki, Hortscience **19** (1984), 507-509; Yasuda, Fuji and Yamaguchi, Plant Cell Physiol. **26** (1985), 595-597; Murashige T. and Skoog F., Physiol. Plant. **15** (1962), 473-497 and Halperin, W. 146 (1964), 408-410, which documents are incorporated herein by way of reference. The buffers may be supplemented by plant hormones such as cytokinines, e.g. 6-benzylaminopurine, or auxins, such as 2,4-dichlorophenoxy acetic acid or mixtures thereof. The skilled artisan will choose the appropriate plant stimulation agent according to his own technical skill. The tissue is the cultivated for a period of time until a primary callus is to be observed on the plant derived tissue.

The present invention is based on the findings that it seemingly sufficient to merely cultivate a plant derived tissue in an induction buffer up to the formation of a primary callus and the plant will nevertheless be capable of grow to a full plant after being stored at cryo-preservation conditions.

The period of time for cultivating the plant derived tissue may depend various parameters, such as the nature of the induction buffer utilized and the plant itself, to which the buffer is applied, but may be within a period of about 6 months, preferably about 2 months. Based on his own technical knowledge the person skilled in the art will choose the appropriate time period for having the explant forming a primary callus.

A preferred induction buffer for cacao is the medium termed A- and the B-medium, respectively, which contains:

A-medium (DKW induction for cocoa)

Macro-Nutrients	*mg/liter*
NH_4NO_3	1416
K_2SO_4	1559
$MgSO_4$, 7 H_2O	740
KH_2PO_4	265
$Ca(NO_3)_2$, $2H_2O$	1968
$CaCl_2$, 2 H_2O	149
Micro-Nutrients	
$Z_n(NO_3)_2$, 6 H_2O	17.00
$MnSO_4$, 1 H_2O	33.50
$CuSO_4$, 5 H_2O	0.25
H_3BO_3	4.80
$Na_2 2MoO_4$, 2 H_2O	0.39
$NiSO_4$, 6 H_2O	0.005
Na_2EDTA	37.3
$FeSO_4$, 7 H_2O	27.8
Vitamins	
Myo-Inositol	100
Nicotinic acid	1
Thiamine HCl(B_1)	2
Amino acids	
Leucine	0.4
Arginine	0.4
Lysine	0.4
Glycine	2.0
Tryptophane	0.2
2,4 dichlorophenoxyacetic acid	1.0
Kinetin	0.25
Sucrose	40 000
Gelrite	3000
pH 5.5	

b) B-medium (DKW expression for cocoa)

Macro-Nutrients	mg/liter
NH_4NO_3	1416
K_2SO_4	1559
$MgSO_4$, 7 H_2O	740
KH_2PO_4	265
$Ca(NO_3)_2$, $2H_2O$	1968
$CaCl_2$, 2 H_2O	149
Micro-Nutrients	
$Zn(NO_3)_2$, 6 H_2O	17.00
$MnSO_4$, 1 H_2O	33.50
$CuSO_4$, 5 H_2O	0.25
H_3BO_3	4.80
$Na_2 2MoO_4$, 2 H_2O	0.39
$NiSO_4$, 6 H_2O	0.005
Na_2EDTA	37.3
$FeSO_4$, 7 H_2O	27.8
Vitamins	
Myo-Inositol	100
Nicotinic acid	1
Thiamine HCl(B_1)	2
Amino acids	
Leucine	0.4
Arginine	0.4
Lysine	0.4
Glycine	2.0
Tryptophane	0.2
Sucrose	40 000
Gelrite	3000

pH 5.5

This primary callus may then be treated by dehydration, pre-freezing and cryo-freezing.

In order to perform the dehydration step any means known in the art to dehydrate the primary explant to an extent that no intracellular ice crystallisation occurs upon the subsequent freezing steps may be envisaged. Such means include e.g. placing explants or pretreated explants or pretreated and encapsulated explants in the air current of a laminar flow cabinet, in a stream of compressed air, in an airtight container together with silica gel or in an airtight container together with various oversaturated salt solutions, that control the relative humidity of the atmsophere.

In a preferred embodiment it has been shown that a two step protocol involving the use of a 0.4 M sucrose solution in the first step and a 1.0 M sucrose solution in the

second step works quite acceptable. The time period required for carrying out steps 1 and 2 vary with respect to the plant to be treated but may well be within a time period of about 10 days, preferably about 4 days.

The pre-freezing step may also be carried out according to techniques well known in the art, such as e.g. at temperatures of from about - 10 to about - 40 °C, most preferably at a temperature of about -25 °C. To this end the cryotubes containing the plant derived tissue to be preserved are preferably placed in a vessel containing an appropriate freezing medium, such as ethanol or isopropanol, at room temperature and the vessel is then transferred into a freezer to slowly cool the sample to the above mentioned temperature. Usually the time period for pre-freezing the sample varies of from 10 to 30 hrs, preferably 15 hrs or more, most preferred about 20 hrs.

Subsequently to the pre-freezing step the tube or vessel containing the induced primary explant may directly be transferred into liquid nitrogen for long term storage.

The primary explants treated accordingly are found to be capable to grow to a full plant after the cryo-preservation.

The invention will now be explained according to the following limiting examples, which are for illustrative purposes only and not to be construed to limit the invention as defined by the appended claims.

EXAMPLES

Example 1 :
Cocoa (genotype a: an eucadorian hybrid genotype termed EET 95)

In this example a process for the in vitro conservation of cocoa genetic resources using cryo-preservation of pieces of flower bud is described.

From cocoa trees growing and flowering in greenhouses young flower buds were collected. Immediately after collection they were sterilised and opened in order to place them onto an induction medium, termed A-medium (supra). After 3 weeks of culture, they were subcultured on a second medium, called B-medium (supra) for

two 3-week culture cycles. Primary embryogenic calli appear during this last culture phase observed by determining the first globular stages among the cells of the primary calli. In the case of creating an embryogenic strain, these calli will be placed on a multiplication medium in order to establish and to maintain a stable proliferation of embryogenic cells.

In this example the cryo-preservation ability of induced explants after 6 weeks of culture (3 weeks on A-medium and 3 weeks on B-medium) was investigated. Therefore, freezing experiments were performed using explants taken before their second transfer onto B-medium.

The freezing protocol used was two step protocol comprising a two-step sucrose pretreatment followed by à two-step cooling phase including a first step in a standard freezer (- 20 °C) before direct immersion in liquid nitrogen. The explants were pretreated first in a 0.4 M sucrose solution for a 3-day period. Then they were transferred into a 1.0 M sucrose solution for a 1-day period before freezing. Pretreated explants are divided into 4 to 6 parts and are then placed into cryotubes (Corning, disposable sterile cryogenic vials, 20 ml, Cat. No. 25704) (in a 1.0 M sucrose solution). The cryotubes are placed in a vessel containing isopropanol, which vessel is transferred to a standard freezer for 20 h. Subsequently, the cryotubes were immersed in liquid nitrogen and stored for 6 months.

For recovery the samples were rapidly thawn by agitation for 2-3 min in a water bath at +40°C.

The samples were then placed on medium A supplemented with 0.8 M sucrose and are then progressively rehydrated by daily subculture on the same medium containing a decreasing sucrose concentration (0.2M sucrose/day) until the sugar concentration reached that of the standard medium (medium A). After about 1 week the samples are transferred to medium B.

The results of the table 1 show that the pre-freezing temperature may influence the success of the cryo-preservation of induced primary explants. Using a mixture of staminodes and etamines as floral piece, the ability to regenerate to plants after freezing in liquid nitrogen was shown.

As control plant tissues without any induction treatment have been used.

Table 1

Effect of the pre-freezing temperature on the viability and regeneration ability of induced primary explants formed from staminodes and etamines after cryo-preservation in liquid nitrogen

			After freezing in LN[1)]
Pre-freezing temperature	Number of pieces	Number of explants	Rate of regrowing explants
Control	60	60	None
- 20 °C	20	110	18
- 25 °C	20	116	44
- 40 °C	20	129	12

1) LN = liquid nitrogen

Example 2:

Cacao (genotype b: brasilian genotype termed MAN15-4)

In the following example the same freezing protocol as in example 1 has been used with the proviso of a change in the pretreatment phase and the cocoa genotype. The plant derived tissues were explants derived from 6-week old staminodes cultures.

The effect of the duration of pretreatment and in particular the incubation period in 0.4M and 1.0M sucrose solution at a pre-freezing temperature of -25°C has been studied.

Table 2

Cryo-preservation of primary explant according to the pretreatment duration

		After freezing in LN[1)]
Pretreatment duration (days) (0.4M / 1.0M sucrose)	number of explants	Rate of regrowing explants
Control	60	non
4d (3d/1d)	39	39
7d (6d / 1d)	42	51
10d (7d / 3d)	38	31

1) LN = liquid nitrogen

From the above it may be derived that the regeneration ability may be improved by subjecting the primary explants a shorter pretreatment and also to a short incubation in 1.0M sucrose solution.

Example 3

In this example the effect of the culture duration on the primary explant before cryo-preservation has been studied. Unless otherwise indicated all steps are as detailed in example 1. The freezing protocol with explants (staminodes) directly placed on pretreatment medium after extraction or after only one week of culture on A-medium has been applied.

Table 3

Cryo-preservation of primary explants on A-medium

Culture conditions of explants	Number of pieces	Number of explants	Rate of regrowing explants
Control	78		none
Without culture on A medium	40	40	40
1 week of culture on A-medium	32	32	62

The results summarized in the above table show that cryo-preserved explants are able to regenerate after freezing without or with a very short culture period on the

induction medium (A-medium). However, a comparison indicates that the longer the cultivating period before freezing, the higher the frequency of regenerating explants.

Example 4

In this example a process for freezing primary explants using the pretreatment - dehydration technique is illustrated. Pretreatment is based on a stepped increase of the sucrose content in the culture medium (0.25M/3d; 0.5M/5d; 0.75M/5d; 1.0M/2d). Then pretreated explants were dehydrated at 43% relative humidity at 24°C in a desiccator. After various dehydration steps the samples are directly immersed in liquid nitrogen.

Table 4

Cryo-preservation of primary explants according to the pretreatment-dehydration protocol.

		After freezing in LN[1)]	
Conditions	Water content (g/100 g dwt)	Number of explants	Rate of growing explants
Control		110	None
Pretreated	185	12	90
Dried 38 hrs	52	42	20
Dried 48 hrs	40	20	30
Dried 62 hrs	28	16	5

1) LN = liquid nitrogen

The results of the above table show that several technical approaches can be developed and optimized for freezing primary explants in liquid nitrogen without essentially altering their ability to regenerate into plants.

Example 5

Coffea canephora (of Vanuatu origin, labelled J21)

Leaf explants are harvested on a tree, then sterilized and placed in culture under aseptic conditions onto an induction medium.

Macro-Nutrients	mg/liter
NH_4NO_3	412
KNO_3	475
$MgSO_4$, 7 H_2O	92.5
KH_2PO_4	85
$CaCl_2$, 2 H_2O	110
Micro-Nutrients (Yasuda, supra)	
$ZnSO_4$, 7 H_2O	4.30
$MnSO_4$, 1 H_2O	6.80
$CuSO_4$, 5 H_2O	0.05
H_3BO_3	3.80
Na_2MoO_4, 2 H_2O	0.125
Na_2EDTA	37.3
$FeSO_4$, 7 H_2O	27.8
Vitamins (from B5 Gamborg medium)	
Meso-Inositol	100.0
Pyridoxine Hcl (B_6)	1.0
Nicotinic acid	1.0
Thiamine HCl(B_1)	10
BAP (6-benzylaminopurine)	1
Sucrose	30 000
Bacto-Difco agar	8 000
pH 5.6	

Vitamins B_5 : Gamborg et al., In vitro, **12** (1976), 473-478).

Primary embryogenic calli appear on the leaf explant during a culture period of 1 to 6 months depending on the genotype and the number of intermediate subcultures.

The freezing protocol used in this example was as described in example 1, above. Leaf explants are pretreated with high sucrose concentration of 1.0M and are then frozen in liquid nitrogen using the above described 2-step cooling method.

The effect of the culture time on the induction medium on the appearance of proliferating calli for both control and cryo-preserved explants has been studied. No subculture has been performed. Table 5 shows that starting from 2 to 6 months primary calli are present on leaf explants at approximately the same rate. After cryo-preservation roughly the same rate of regrowing calli is observed.

Table 5

Cryo-preservation of induced leaf explant of Coffea canephora

Culture duration of the leaf explant before freezing	Control	After cryo-preservation
	Rate of primary calli	Rate of primary calli
2 months	95	52
4 months	95	54
6 months	91	48

Example 6

Two other Coffea canephora genotypes (of Vanuatu orogin, labelled NC8 and NC 109, repsectively) have been investigated. Table 6 shows a high level of regrowth after cryo-preservation and the effective production of plantlets obtained from primary embryos selected on the new embryogenic proliferation after freezing.

Table 6

Cryo-preservation of induced leaf explant of Coffea canephora

Genotypes	Cryo-preserved plants		Conversion rate of primary embryos formed from cryo-preserved explants	
	Rate of regrowing calli	Rate of embryogenic calli	Pretreated	Cryo-preserved
A	84	50	30	22
B	100	75	30	25

Example 7

Carrot

In the case of carrot somatic embryogenesis, most of the embryogenic strains are initiated from hypocotyl segments of a germinating seed. These pieces of hypocotyl have been obtained by cultivation on the following induction medium:

Medium used as preferred induction buffer for *carrot (Daucus carota L.):*

Macro-Nutrients	mg/liter
NH_4NO_3	1650.00
KNO_3	1900.00
$MgSO_4$, 7 H_2O	370.0
KH_2PO_4	170.0
$CaCl_2$, 2 H_2O	440.0
Micro-Nutrients (Murashige and Skoog, 1962)	
$ZnSO_4$, 7 H_2O	10.600
$MnSO_4$, 1 H_2O	16.900
$CuSO_4$, 5 H_2O	0.025
H_3BO_3	6.200
Na_2MoO_4, 2 H_2O	0.250
$CoCl_2$, 6 H_2O	0.025
KCl	0.830
Na_2EDTA	37.3
$FeSO_4$, 7 H_2O	27.8
Vitamins (Halperin, 1964)	
Adenine	2.0
Thiamine HCl(B_1)	5.0
Nicotinic acid	5.0
Thiamine HCl(B_1)	5.0
2,4-D	0.1
Sucrose	20 000
Bacto-Difco agar	8 000
pH 5.8	

for a time period of about 3 to 4 weeks. After observing the formation of primary calli, usually after 3 weeks the explants are treated as described below.

A freezing protocol based on first inducing desiccation tolerance and second dehydrating under controlled relative humidity was applied with a final direct immersion in liquid nitrogen.

Desiccation tolerance is induced by culture of hypocotyl explants on the above induction medium supplemented with 0.4M-0.5M sucrose concentration.

Dehydration is performed by equilibration of the tissues under controlled relative humidity in the range of 43 to 11% at 24°C during 7 days. Subsequently the dried explants are directly immersed in liquid nitrogen.

Table 7

Cryo-preservation of carrot hypocotyl using a desiccation method

Starting conditions		After drying under 43 % and freezing in LN	
	Number of explants	Rates of surviving explants	Rates of embryogenic explants
Without culture on induction medium	30	23	13
After 3 week culture on induction medium	30	66	33

It could be shown that the initial culture phase on the induction medium before freezing improves the rate of success of the method.

From the examples it could be clearly seen that cryo-preservation of primary explants capable to regenerate plants was successfully performed with cocoa , coffee and carrot species. The primary explants used are pieces of flower bud, pieces of leaves and hypocotyl segments, respectively. Induction of freezing tolerance using an hardening-off treatment in presence of high sucrose concentrations has exemplarily been used. Various freezing methods can be used, i.e. the classical method with some simplifications and a pre-freezing step at preferably about –25°C or a partial dehydration before freezing for species known to be desiccation sensitive (cacao, coffee) and a desiccation before freezing for carrot which is considered as desiccation tolerant species.

According to the present invention the time period to introduce an accession in a gene bank could enormously be reduced while preserving the capability to regenerate into a plants after cryo-preservation. The process described allows to envisage long-term storage of genetic resources for large number of accessions without the drawbacks inherent to the establishment and maintenance of in vitro culture.

CLAIMS

1 A process for the cryo-preservation of plant tissues comprising the steps of dehydrating, pre-freezing and cryo-freezing plant derived tissue, wherein the plant tissue to be cryo-preserved is a primary explant that has been subjected an induction treatment for regeneration.

2. The process for the cryo-preservation according to claim 1, wherein the plant tissue is a primary explant capable to regenerate buds.

3. The process for the cryo-preservation according to claim 1, wherein the plant tissue is a primary explant capable to regenerate embryos.

4. The process for the cryo-preservation according to claim 1 or 2, wherein the plant tissue utilised is derived from a plant belonging to the genus theobroma cacao, Coffea canephora, Coffea arabica or Daucus carota.

5. The process for the cryo-preservation according to claim 4, wherein the plant tissue utilized is derived from Coffea canephora or Coffea arabica

6. The process according to claim 4, wherein the plant tissue utilized is derived from cacao.

7. The process according to claim 4, wherein the plant tissue utilized is derived from carrots.

8. The process according to any of the preceding claims, wherein the dehydration step involves the use of a two step incubation of the explants in an medium containing 0.4 M sucrose in the first step and in a medium containing 1M sucrose in the second step.

SUMMARY

The present invention pertains to an improved process for the cryo-preservation of plants, especially for the direct cryo-preservation of plant tissues, the process comprising the steps of dehydrating, pre-freezing and cryo-freezing plant derived tissue, wherein the plant tissue to be cryo-preserved is a primary explant that has been subjected an induction treatment for regeneration.